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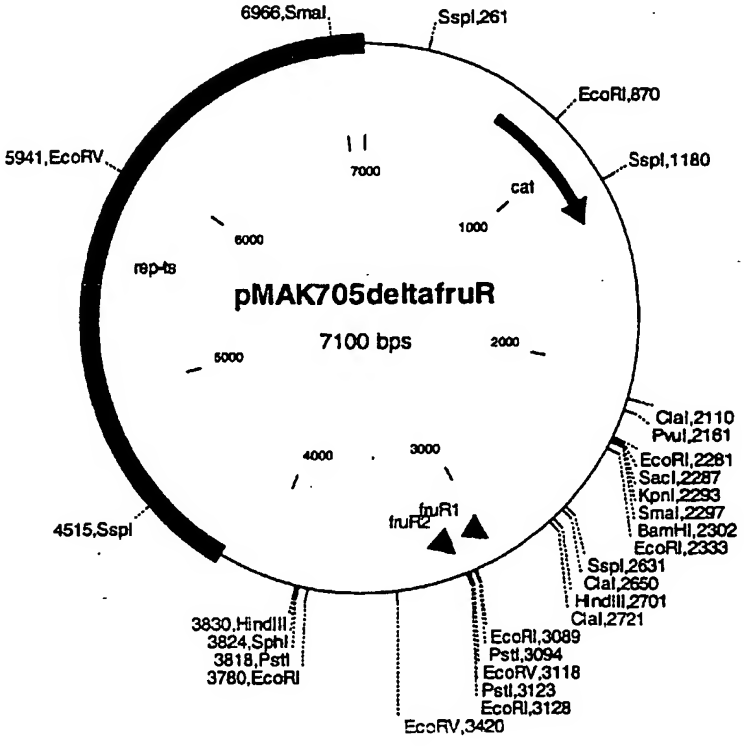
(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTERIACEAE THAT CONTAIN AN ATTENUATED FRUR GENE



(57) Abstract: The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the fruR gene or nucleotide sequences coding therefor are attenuated, in particular are switched off, b) enrichment of the L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the L-amino acid.

WO 02/081698 A2

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Process for the Production of L-Amino Acids using Strains of the Family Enterobacteriaceae that contain an Attenuated fruR Gene

Field of the Invention

The present invention relates to a process for the enzymatic production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae in which the fruR gene is attenuated.

Prior Art

L-amino acids, in particular L-threonine, are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry, and most especially in animal nutrition.

It is known to produce L-amino acids by fermentation of strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. On account of their great importance efforts are constantly being made to improve processes for producing the latter. Process improvements may relate to fermentation technology measures, such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during the fermentation, or the working-up to the product form, for example by ion exchange chromatography, or the intrinsic performance properties of the microorganism itself.

Methods comprising mutagenesis, selection and mutant choice are employed in order to improve the performance properties of these microorganisms. In this way strains are obtained that are resistant to antimetabolites, such as for example the threonine analogue α-amino-β-hydroxyvaleric acid (AHV) or are auxotrophic for regulatorily important metabolites, and that produce L-amino acids such as for example L-

Methods of recombinant DNA technology have also been used for some years in order to improve strains of the family Enterobacteriaceae producing L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating their effect on production.

Object of the Invention

The object of the invention is to provide new measures for the improved enzymatic production of L-amino acids, in particular L-threonine.

Summary of the Invention

The invention provides a process for the enzymatic production of L-amino acids, in particular L-threonine, using microorganisms of the family Enterobacteriaceae that in particular already produce L-amino acids and in which the nucleotide sequence coding for the fruR gene is attenuated.

Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned hereinafter, this is understood to mean one or more amino acids including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-threonine is particularly preferred.

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by using for example a weak promoter or a gene or allele that codes for a corresponding enzyme with a low activity and/or that inactivates the

corresponding enzyme (protein) or gene, and optionally combining these measures.

By means of these attenuation measures the activity or concentration of the corresponding protein is generally reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild type protein, or the activity or concentration of the protein in the initial microorganism.

The process is characterized in that the following steps are carried out:

a) fermentation of microorganisms of the family Enterobacteriaceae in which the fruR gene is attenuated,

b) enrichment of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the family Enterobacteriaceae, and

c) isolation of the desired L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or parts thereof remain in the product.

The microorganisms that are the subject of the present invention can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. The microorganisms are members of the family Enterobacteriaceae selected from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. In the case of the genus Escherichia the species Escherichia coli may in particular be mentioned, and in the case of the genus Serratia the species Serratia marcescens may in particular be mentioned.

Suitable strains of the genus Escherichia, in particular those of the species Escherichia coli, that produce in particular L-threonine, include for example:

Escherichia coli TF427
Escherichia coli H4578
Escherichia coli KY10935
Escherichia coli VNIIgenetika MG442
Escherichia coli VNIIgenetika M1
Escherichia coli VNIIgenetika 472T23
Escherichia coli BKIIM B-3996
Escherichia coli kat 13
Escherichia coli KCCM-10132

Suitable strains of the genus Serratia, in particular of the species Serratia marcescens, that produce L-threonine include for example:

Serratia marcescens HNr21
Serratia marcescens TLr156
Serratia marcescens T2000

Strains of the family of Enterobacteriaceae producing L-threonine preferably have, *inter alia*, one or more of the genetic or phenotype features selected from the following group: resistance to α-amino-β-hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α-methylserine, resistance to diaminosuccinic acid, resistance to α-aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues such as for example valine hydroxamate, resistance to purine analogues such as for example 6-dimethylaminopurine, need for L-methionine, optionally partial and compensatable need for L-isoleucine, need for meso-diaminopimelic acid, auxotrophy with regard to threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid,

resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, optionally ability to utilise sucrose, enhancement of the threonine operon, enhancement of homoserine dehydrogenase, I-aspartate kinase I, preferably of the feedback-resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feedback-resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feedback-resistant form, enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

It has now been found that microorganisms of the family Enterobacteriaceae after attenuation, in particular after switching off the fruR gene, produce L-amino acids, in particular L-threonine, in an improved way.

The nucleotide sequences of the Escherichia coli genes belong to the prior art and may also be obtained from the genome sequence of Escherichia coli published by Blattner et al. (Science 277, 1453 - 1462 (1997)).

The fruR gene is described *inter alia* by the following data:

Designation:	Fructose repressor
EC-No.:	-
Reference:	Jahreis et al., Molecular and General Genetics 226, 332-336 (1991)
Accession No.:	AE000118

Comment: The fruR gene is also designated in the prior art as cra gene.

Apart from the described fruR gene, alleles of the gene may be used that result from the degeneracy of the genetic code or from functionally neutral sense mutations, the activity of the protein not being substantially altered.

In order to achieve an attenuation the expression of the gene or the catalytic properties of the enzyme proteins may for example be reduced or switched off. Optionally both measures may be combined.

The gene expression may be reduced by suitable culture conditions, by genetic alteration (mutation) of the signal structures of the gene expression, or also by antisense-RNA techniques. Signal structures of the gene expression are for example repressor genes, activator genes, operators, promoters, attenuators, ribosome-binding sites, the start codon and terminators. The person skilled in the art may find relevant information in, *inter alia*, articles by Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), by Carrier and Keasling (Biotechnology Progress 15, 58-64 (1999)), Franch and Gerdes (Current Opinion in Microbiology 3, 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change or reduction of the catalytic properties of enzyme proteins are known from the prior art. As examples there may be mentioned the works by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences, USA 95, 5511-5515 (1998)), Wente and Schachmann (Journal of Biological Chemistry 266, 20833-

20839 (1991)). Descriptive overviews may be obtained from known textbooks on genetics and molecular biology, such as for example that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations include transitions, transversions, insertions and deletions. Depending on the action of the amino acid exchange on the enzyme activity, one speaks of missense mutations or nonsense mutations. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations, which in turn lead to the incorporation of false amino acids or the premature termination of a translation. If as a result of the mutation a stop codon is formed in the coding region, this also leads to a premature termination of the translation. Deletions of several codons typically lead to a complete disruption of the enzyme activity. Details regarding the production of such mutations belong to the prior art and may be obtained from known textbooks on genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations in the genes such as for example deletion mutations may be incorporated by gene and/or allele exchange in suitable strains.

A conventional method is the method of gene exchange by means of a conditionally replicating pSC101 derivate pMAK705 described by Hamilton et al. (Journal of Bacteriology 171, 4617 - 4622 (1989)). Other methods described in the prior art, such as for example that of Martinez-Morales et al. (Journal of Bacteriology 181, 7143-7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182, 842-847 (2000)) may likewise be used.

It is also possible to transfer mutations in the respective genes or mutations relating to the expression of the relevant genes, by conjugation or transduction into various strains.

Furthermore for the production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae it may be advantageous in addition to the attenuation of the fruR gene also to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide-adenine-dinucleotide phosphate.

The term "enhancement" describes in this connection the raising of the intracellular activity of one or more enzymes or proteins in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes, using a strong promoter or a gene that codes for a corresponding enzyme or protein having a high activity, and optionally by combining these measures.

By means of the aforementioned enhancement measures, in particular overexpression, the activity or concentration of the corresponding protein is in general raised by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at most up to 1000% or 2000% referred to that of the wild type protein and/or the activity or concentration of the protein in the initial microorganism.

Thus, one or more of the genes selected from the following group may for example by simultaneously enhanced, in particular overexpressed:

- the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),

- the pyc gene coding for pyruvate carboxylase (DE-A-19 831 609),
- the pps gene coding for phosphoenol pyruvate synthase (Molecular and General Genetics 231:332 (1992)),
- the ppc gene coding for phosphoenol pyruvate carboxylase (Gene 31:279-283 (1984)),
- the genes pntA and pntB coding for transhydrogenase (European Journal of Biochemistry 158:647-653 (1986)),
- the gene rhtB imparting homoserine resistance (EP-A-0 994 190),
- the mqo gene coding for malate:quinone oxidoreductase (DE 100 348 33.5),
- the gene rhtC imparting threonine resistance (EP-A-1 013 765), and
- the thrE gene of Corynebacterium glutamicum coding for threonine export (DE 100 264 94.8).

The use of endogenous genes is in general preferred. The term "endogenous genes" or "endogenous nucleotide sequences" is understood to mean the genes or nucleotide sequences present in the population of a species.

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition to the attenuation of the fruR gene also to attenuate, in particular to switch off or reduce the expression of one or more of the genes selected from the following group:

- the tdh gene coding for threonine dehydrogenase (Ravnikar and Somerville, Journal of Bacteriology 169, 4716-4721 (1987)),

- the mdh gene coding for malate dehydrogenase (E.C. 1.1.1.37) (Vogel et al., Archives in Microbiology 149, 36-42 (1987)),

- the gene product of the open reading frame (orf) yjfA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),

- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),

- the pckA gene coding for the enzyme phosphoenol pyruvate carboxykinase (Medina et al. (Journal of Bacteriology 172, 7151-7156 (1990)),

- the poxB gene coding for pyruvate oxidase (Grabau and Cronan (Nucleic Acids Research 14 (13), 5449-5460 (1986)),

- the aceA gene coding for isocitrate lyase (EC-No.: 4.1.3.1) (Matsuoko and McFadden; Journal of Bacteriology 170, 4528-4536 (1988) and Accession No.: AE000474), and

- the dgsA gene coding for the regulator of the phosphotransferase system (Hosono et al., Bioscience, Biotechnology and Biochemistry 59, 256-261 (1995) and Accession No.: AE000255)

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition to the attenuation of the fruR gene also to switch off undesirable secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention may be cultivated in a batch process (batch cultivation), in a

fed batch process (feed process) or in a repeated fed batch process (repetitive feed process). A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Brunswick/ Wiesbaden, 1994)).

The culture medium to be used must appropriately satisfy the requirements of the respective strains. Descriptions of culture media of various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

As carbon sources, sugars and carbohydrates such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats such as for example soya bean oil, sunflower oil, groundnut oil and coconut oil, fatty acids such as for example palmitic acid, stearic acid and linoleic acid, alcohols such as for example glycerol and ethanol, and organic acids such as for example acetic acid, may be used. These substances may be used individually or as a mixture.

As nitrogen source, organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, maize steep liquor, soya bean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate may be used. The nitrogen sources may be used individually or as a mixture.

As phosphorus source, phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts may be used. The culture medium must furthermore contain salts of metals,

such as for example magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth promoters such as amino acids and vitamins may be used in addition to the aforementioned substances. Apart from these, suitable precursors may be added to the culture medium. The aforementioned starting substances may be added to the culture in the form of a single batch or may be metered in in an appropriate manner during the cultivation.

In order to regulate the pH of the culture basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds such as phosphoric acid or sulfuric acid are used as appropriate. In order to control foam formation antifoaming agents such as for example fatty acid polyglycol esters may be used. In order to maintain the stability of plasmids, suitable selectively acting substances, for example antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures such as for example air are fed into the culture. The temperature of the culture is normally 25°C to 45°C, and preferably 30°C to 40°C. Cultivation is continued until a maximum amount of L-amino acids (or L-threonine) has been formed. This target is normally achieved within 10 hours to 160 hours.

The L-amino acids may be analyzed by anion exchange chromotography followed by ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention can be used for the enzymatic production of L-amino acids, such as for example L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

A pure culture of the Escherichia coli K-12 strain DH5α/pMAK705 was filed as DSM 13720 on 8 September 2000 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) according to the Budapest Convention.

The present invention is described in more detail hereinafter with the aid of examples of implementation.

The isolation of plasmid DNA from Escherichia coli as well as all techniques for the restriction, ligation, Klenow treatment and alkaline phosphatase treatment are carried out according to Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). The transformation of Escherichia coli is, unless otherwise described, carried out according to Chung et al. (Proceedings of the National Academy of Sciences of the United States of America, USA (1989) 86: 2172-2175).

The incubation temperature in the production of strains and transformants is 37°C. In the gene exchange process according to Hamilton et al, temperatures of 30°C and 44°C are used.

Example 1

Construction of the deletion mutation of the fruR gene

Parts of the gene regions and parts of the 5`- and 3`-region of the fruR gene from Escherichia coli K12 lying upstream and downstream of the fruR gene are amplified using the polymerase chain reaction (PCR) as well as synthetic oligonucleotides. Starting from the nucleotide sequence of the fruR gene and sequences in E. coli K12 MG1655 DNA (SEQ ID No. 1, Accession Number AE000118) lying upstream and downstream, the following PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

fruR'5'-1: 5' - ATGAATCAGGCGCGTTATCC - 3' (SEQ ID No. 3)

fruR'5'-2: 5' - TTGTCGCTCACACGGTATTG - 3' (SEQ ID No. 4)

fruR'3'-1: 5' - AGCGTGTGCTGGAGATTGTC - 3' (SEQ ID No. 5)

fruR'3'-2: 5' - AGCCAGTCACAAGGCATACC - 3' (SEQ ID No. 6)

The chromosomal E. coli K12 MG1655 DNA used for the PCR is isolated according to the manufacturer's instructions using "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A ca. 750 bp large DNA fragment from the 5' region of the fruR gene region (designated fruR1) and a ca. 650 bp large DNA fragment from the 3` region of the fruR gene region (designated as fruR2) may be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with the taq-DNA-polymerase (Gibco-BRL, Eggenstein, Germany). The PCR products are ligated according to the manufacturer's instructions in each case with the vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, Netherlands) and transformed in the E. coli strain TOP10F'. The selection of plasmid-carrying cells is carried out on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA isolation the vector pCR2.1TOPOfruR2 is cleaved with the restriction enzyme NotI and the supernatant 3`-ends are treated with Klenow enzyme. After the restriction with the enzyme SpeI the fruR2 fragment is separated in 0.8% agarose gel and isolated using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After the plasmid DNA isolation, the vector pCR2.1TOPOfruR1 is cleaved with the enzymes EcoRV and XbaI and ligated with the isolated fruR2 fragment. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA isolation those plasmids in which the mutagenic DNA sequence illustrated in SEQ ID No. 7 is present in cloned form are detected by control cleavage with the enzymes

HindIII, EcoRV and PvuI. One of the plasmids is designated pCR2.1TOPOΔfruR.

Example 2

Construction of the exchange vector pMAK705ΔfruR

The fruR allele described in Example 1 is isolated from the vector pCR2.1TOPOΔfruR after restriction with the enzyme Eco0109I, treatment of the supernatant 3`-ends with Klenow enzyme, restriction with the enzyme BamHI and separation in 0.8% agarose gel, and ligated with the plasmid pMAK705 (Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622) that has been digested with the enzymes HincII and BamHI. The ligation batch is transformed in DH5α and plasmid-carrying cells are selected on LB agar to which 20 µg/ml chloramphenicol had been added. Successful cloning is detected after plasmid DNA isolation and cleavage with the enzymes HindIII, BamHI, EcoRV, ScaI and SpeI. The resultant exchange vector pMAK705ΔfruR (= pMAK705deltafruR) is shown in Fig. 1.

Example 3

Site-specific mutagenesis of the fruR gene in the E. coli strain MG442

The E. coli strain MG442 producing L-threonine is described in patent specification US-A- 4,278,765 and is filed as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

For the exchange of the chromosomal fruR gene by the plasmid-coded deletion construct, MG442 is transformed with the plasmid pMAK705ΔfruR. The gene exchange is carried out by the selection process described by Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR

Protocols. A guide to methods and applications, Academic Press) with the following oligonucleotide primers:

fruR'5'-1: 5' - ATGAATCAGGCGCGTTATCC - 3' (SEQ ID No. 3)

fruR'3'-2: 5' - AGCCAGTCACAAGGCATACC - 3' (SEQ ID No. 6)

After exchange has been carried out the form of the ΔfruR allele illustrated in SEQ ID No. 8 is present in MG442. The resultant strain is designated MG442ΔfruR.

Example 4

Production of L-threonine using the strain MG442ΔfruR

MG442ΔfruR is cultivated on minimal medium having the following composition: 3.5 g/l $Na_2HPO_4 \cdot 2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l glucose and 20 g/l agar. The formation of L-threonine is checked in batch cultures of 10 ml that are contained in 100 ml Erlenmeyer flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose are inoculated and incubated for 16 hours at 37°C and 180 rpm in an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl of this preculture are reinoculated in 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4 \cdot 7H_2O$, 0.03 g/l $FeSO_4 \cdot 7H_2O$, 0.018 g/l $MnSO_4 \cdot 1H_2O$, 30 g/l $CaCO_3$ and 20 g/l glucose) and incubated for 48 hours at 37°C. After incubation the optical density (OD) of the culture suspension is measured with an LP2W photometer from the Dr. Lange company (Dusseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of formed L-threonine is then determined in the sterile-filtered culture supernatant using an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany)

- BglII: restriction endonuclease from Bacillus globigii
- ClaI: restriction endonuclease from Caryphanon latu
- EcoRI: restriction endonuclease from Escherichia col
- EcoRV: restriction endonuclease from Escherichia col
- HindIII: restriction endonuclease from Haemophilus influenzae
- KpnI: restriction endonuclease from Klebsiella pneumoniae
- PstI: restriction endonuclease from Providencia stuartii
- PvuI: restriction endonuclease from Proteus vulgari
- SacI: restriction endonuclease from Streptomyces achromogenes
- SalI: restriction endonuclease from Streptomyces albus
- SmaI: restriction endonuclease from Serratia marcescens
- SphI: restriction endonuclease from Streptomyces phaeochromogenes
- SspI: restriction endonuclease from Sphaerotilus species
- XbaI: restriction endonuclease from Xanthomonas badrii
- XhoI: restriction endonuclease from Xanthomonas holcicola

What is Claimed is:

1. Process for the production of L-amino acids, in particular L-threonine, wherein the following steps are carried out:

   a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the fruR gene or nucleotide sequences coding therefor are attenuated, in particular are switched off,

   b) enrichment of the L-amino acid in the medium or in the cells of the microorganisms, and

   c) isolation of the L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or portions thereof remain in the product.

2. Process according to claim 1, wherein microorganisms are used in which in addition further genes of the biosynethesis pathway of the desired L-amino acid are enhanced.

3. Process according to claim 1, wherein microorganisms are used in which the metabolic pathways that reduce the formation of the desired L-amino acid are at least partially switched off.

4. Process according to claim 1, wherein the expression of the polynucleotide(s) that codes/code for the fruR gene is attenuated, in particular is switched off.

5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide fruR codes are reduced.

6. Process according to claim 1, wherein for the production of L-amino acids microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is enhanced, in particular overexpressed:

6.1 the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,

6.2 the pyc gene coding for pyruvate carboxylase,

6.3 the pps gene coding for phosphoenol pyruvate synthase,

6.4 the ppc gene coding for phosphoenol pyruvate carboxylase,

6.5 the genes pntA and pntB coding for transhydrogenase,

6.6 the gene rhtB imparting homoserine resistance,

6.7 the mqo gene coding for malate:quinone oxidoreductase,

6.8 the gene rhtC imparting threonine resistance, and

6.9 the thrE gene coding for threonine export.

7. Process according to claim 1, wherein for the production of L-amino acids microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is attenuated, in particular switched off, or the expression is reduced:

7.1 the tdh gene coding for threonine dehydrogenase,

7.2 the mdh gene coding for malate dehydrogenase,

7.3 the gene product of the open reading frame (orf) yjfA,

7.4 the gene product of the open reading frame (orf) ytfP,

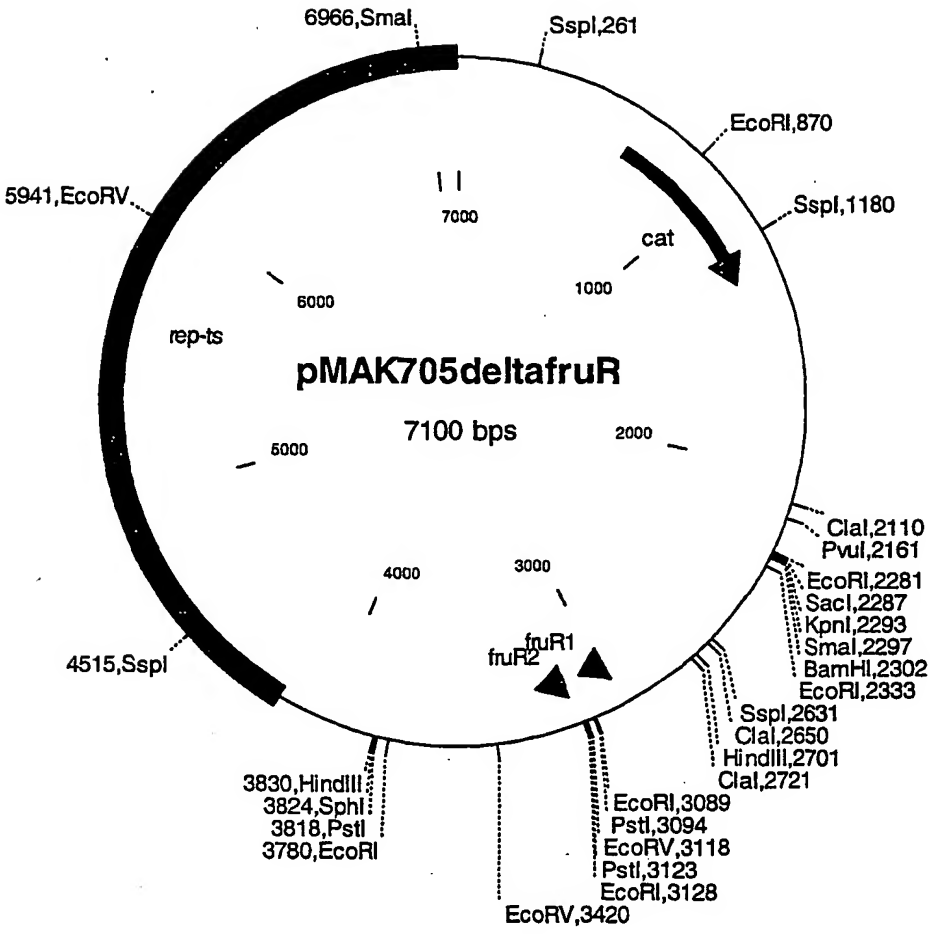
7.5 the pckA gene coding for phosphoenol pyruvate carboxykinase,

7.6 the poxB gene coding for pyruvate oxidase,

7.7 the aceA gene coding for isocitrate lyase,

7.8 the dgsA gene coding for the regulator of the phosphotransferase system.

Fig. 1: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SEQUENCE LISTING

<110> Degussa AG

<120> Process for the production of L-amino acids using strains of the family Enterobacteriaceae containing an attenuated fruR gene

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PCT

Original (for SUBMISSION) - printed on 05.03.2002 09:02:18 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited *Microorganism(s) or Other Biological* Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2002)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	020005 BT

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	13
1-2	line	1-5
1-3	*Identification of Deposit*	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	08 September 2000 (08.09.2000)
1-3-4	Accession Number	DSMZ 13720
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	yes
0-4-1	Authorized officer	C.A.J.A. PASCHE

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa-Hüls AG
Kantstr. 2
33790 Halle

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: DH5α/pMAK705	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(X) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2000-09-08 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Dagmar Fricke Date: 2000-09-12

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page, 01/99)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa-Hüls AG
Kantstr. 2
33790 Halle

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa-Hüls AG Kantstr. 2 Address: 33790 Halle	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720 Date of the deposit or the transfer[1]: 2000-09-08

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2000-09-08 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 2000-09-12

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 01.96

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number
WO 02/081698 A3

(51) International Patent Classification[7]: C12P 13/08, 13/04 // (C12P 13/08, C12R 1:19)

(21) International Application Number: PCT/EP02/02420

(22) International Filing Date: 6 March 2002 (06.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
101 16 518.8 3 April 2001 (03.04.2001) DE

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors: RIEPING, Mechthild; Mönkebergstrasse 1, 33619 Bielefeld (DE). HERMANN, Thomas; Zirkonstrasse 8, 33739 Bielefeld (DE).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

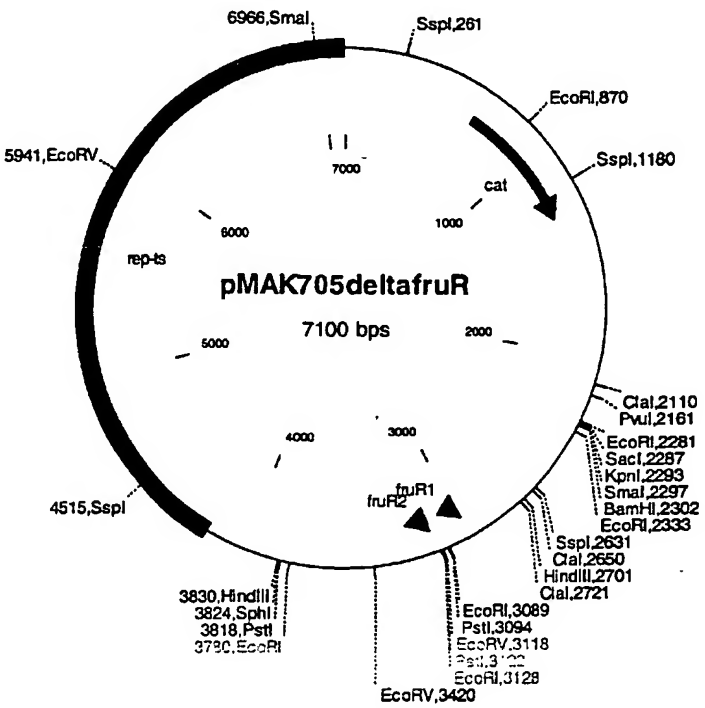
(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report
— with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description

(88) Date of publication of the international search report:
30 October 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTERIACEAE THAT CONTAIN AN ATTENUATED FRUR GENE



(57) Abstract: The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the fruR gene or nucleotide sequences coding therefor are attenuated, in particular are switched off, b) enrichment of the L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the L-amino acid.

WO 02/081698 A3

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 02/02420

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/08 C12P13/04 //(C12P13/08,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99 53035 A (ALTMAN ELLIOT ;GOKARN RAVI R (US); EITEMAN MARK A (US); UNIV GEORG) 21 October 1999 (1999-10-21) page 5, line 20-24 examples 4,7,9,10 claims 41,49 figures 1,4 ---	1-7
Y	RAMSEIER T M: "Cra and the control of carbon flux via metabolic pathways." RESEARCH IN MICROBIOLOGY, vol. 147, no. 6-7, July 1996 (1996-07), pages 489-493, XP002241023 ISSN: 0923-2508 the whole document figure 1 ---	1-7
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
20 May 2003	02/06/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	van de Kamp, M

Form PCT/ISA/210 (second sheet) (July 1992)

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